Glucose controls sex-related growth rate differences of bovine embryos produced *in vitro*

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The objective of this study was to determine whether the presence of glucose in the culture medium causes more growth of male embryos than of female embryos in cows. Oocytes matured and fertilized *in vitro* were cultured from 24 to 48 h after insemination in CR1aa medium with or without 5.56 mmol glucose l^{-1} . At the end of culture the mean numbers of cells of the cleaved embryos were 5.07 (± 0.16) in glucose-free medium and 5.29 (± 0.17) in medium with glucose ($P=0.86$). Without glucose in the medium, the least square mean was 0.22 cells higher in males than in females. This difference was not significant ($P=0.44$). However, in the presence of glucose this difference was 1.25 cells in favour of males ($P=0.0001$). The possibility that the sex difference is linked with growth stimulating effects of oxygen radicals is discussed.

Introduction

Several studies in mice (Tsunoda *et al.*, 1985; Burgoyne, 1993; Valdivia *et al.*, 1993; Zwingman *et al.*, 1993; Peippo and Bredbacka, 1995) and cattle (Avery *et al.*, 1991, 1992; Xu *et al.*, 1992; Yadav *et al.*, 1993) have suggested that male embryos develop faster than female embryos. Although Bondioli *et al.* (1989) reported a biased sex ratio in cow embryos produced *in vivo*, the growth difference appears to be enhanced by, if not limited to, the *in vitro* environment (Callesen *et al.*, 1995; Peippo and Bredbacka, 1995). In cows there is evidence that this difference is initiated by the time of the first cleavage (Dominko and First, 1993; Yadav *et al.*, 1993).

The reason for the tendency of males to develop faster is unknown. It has been suggested that the growth differences are caused by Y-chromosomal growth factors (Avery *et al.*, 1989; Xu *et al.*, 1992; Burgoyne, 1993; Ray *et al.*, 1995) but the role of X-chromosomal enzymes has also been considered (Rieger, 1992; Burgoyne and Thornhill, 1995; Peippo and Bredbacka, 1995). During early development, both X chromosomes in females are active, and one becomes inactivated during the blastocyst stage (Kratzer and Gartler, 1978; de la Fuente *et al.*, 1993). Consequently, in mice at least, the expression of X-chromosomal genes encoding, for example, hypoxanthine phosphoribosyl transferase (HPRT) and glucose-6-phosphate dehydrogenase (G6PDH) is approximately twice as high in females than in males between the time of genome activation and X chromosome inactivation (Epstein *et al.*, 1978). These enzymes apparently play a role in decreasing intracellular concentrations of oxygen radicals (Rieger, 1992). Before X chromosome inactivation, these concentrations therefore may be lower than in males.

The present study is based on the hypothesis that the growth rate difference between the sexes is caused by the

increased expression of G6PDH or HPRT in female embryos in the presence of glucose, resulting in a decreased production of oxygen radicals. This hypothesis was tested by culturing fertilized oocytes *in vitro* in media with or without glucose from 24 to 48 h after insemination. According to our hypothesis, the faster cleavage of male embryos is enhanced by glucose.

Materials and Methods

In vitro maturation

Cattle ovaries were obtained from an abattoir and transported to the laboratory at room temperature in 0.9% (w/v) NaCl supplemented with 100 iu penicillin ml^{-1} and 100 μg streptomycin ml^{-1} . The oocytes were aspirated from follicles with a diameter of 3–8 mm. Only oocytes surrounded by cumulus cells were selected for maturation. Oocyte–cumulus complexes were washed twice in TALP–Hepes (Parrish *et al.*, 1988). Hepes was obtained from Sigma (St Louis, MO). The maturation medium was TCM199 (Sigma) supplemented with 0.25 mmol sodium pyruvate l^{-1} , 2 mmol L-glutamine l^{-1} , 100 iu penicillin ml^{-1} , 100 μg streptomycin ml^{-1} , 2 μg FSH ml^{-1} (USDA-oFSH-18; USDA, Beltsville, MD), 10 μg LH ml^{-1} (USDA-bLH-B-6; USDA, Beltsville, MD), 1 μg oestradiol ml^{-1} (Sigma), 5% (v/v) fetal calf serum and 5% (v/v) oestrous cow serum. In each 500 μl drop of medium, 40–50 oocyte–cumulus complexes were matured for 24 h under 5% CO_2 in air at 39°C.

In vitro fertilization

After maturation, oocyte–cumulus complexes were washed in TALP–Hepes and transferred to 100 μl fertilization drops covered with embryo tested mineral oil (Sigma). The fertilization medium was fert-TALP (Parrish *et al.*, 1988)

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supplemented with 2 μg heparin ml^{-1} , 20 μmol penicillamine l^{-1} , 10 μmol hypotaurine l^{-1} and 1 μmol adrenaline l^{-1} (Sigma). Frozen-thawed spermatozoa were washed twice in sperm-TALP (Parrish *et al.*, 1988) and 1.5×10^6 spermatozoa ml^{-1} was added into each fertilization drop. After 24 h fertilization at 39°C in 5% CO_2 :95% air, the cumulus cells were removed by vortexing.

In vitro culture

The oocytes were washed in glucose-free CR1aa-medium (Rosenkrans and First, 1991) and placed randomly into one of the two culture media: CR1aa (without glucose) and CR1aa supplemented with 5.56 mmol glucose l^{-1} . About 30–60 embryos were cultured in each 500 μl drop of medium covered with mineral oil at 39°C in a humidified atmosphere of 5% CO_2 in air. After culture for 24 h the number of cells of each embryo was recorded. Only cleaved embryos were used for further analysis.

Sexing of embryos using PCR

At the end of culture, the sex of individual cleaved embryos was determined using PCR (Saiki *et al.*, 1988). Accessory spermatozoa were removed by removing the zonae pellucidae by brief exposure to Tyrode's solution (pH 1.8–2.0) and washing three times in 0.5 ml PBS with 6 mg polyvinylpyrrolidone ml^{-1} (Sigma). With minor modifications, the embryos were sexed as described by Bredbacka and Peippo (1992), using primers designed to amplify the Y-chromosomal locus, ZFY, and its X-chromosomal homologue, ZFX. Each embryo was placed in 1 μl PBS + polyvinylpyrrolidone into a 0.5 ml reaction tube filled with 9 μl lysis buffer consisting of DynaZyme[™] buffer (Finnzymes, Espoo, Finland), dNTPs, MgCl_2 and 0.1 mg proteinase K ml^{-1} (Boehringer Mannheim, Mannheim, Germany). After lysis for 30 min at 37°C, the mixture was overlaid with 25 μl DNase-free mineral oil (Sigma) and then proteinase K was inactivated at 98°C for 10 min. During the 3 min denaturation step of the first PCR cycle, 15 μl MgCl_2 -free DynaZyme[™] buffer (including the primers and the polymerase) was added to each tube. The final 25 μl reaction mixture consisted of 50 mmol KCl l^{-1} , 1.5 mmol MgCl_2 l^{-1} , 10 mmol Tris-HCl l^{-1} (pH 8.8), 0.01% gelatin (w/v), 0.1% Triton X-100 (v/v), 0.2 mmol dNTPs l^{-1} , 1.5 U of DynaZyme[™] polymerase (Finnzymes) and 10 pmol of each primer. The amplification was carried out using an MJ Research Minicycler[™] and consisted of an initial denaturation for 3 min at 94°C followed by 50 cycles of 60 s at 94°C, 50 s at 54°C and 30–60 s at 72°C. Finally, the samples were incubated for 5 min at 72°C.

After PCR, a 10 μl sample from each tube was added to a tube containing 10 μl digestion buffer (100 mmol NaCl l^{-1} , 18 mmol MgCl_2 l^{-1} , 5 U endonuclease PstI, 10 mmol Tris-HCl l^{-1} , pH 7.5). After digestion for 1–2 h the Y-chromosomal homologue could be digested into fragments of 344 bp and 103 bp, whereas the X-chromosomal homologue remained at 445 bp. Consequently, a male sample was characterized by the presence of all three fragments, whereas only the longest fragment could be observed in female samples. The fragment

lengths were observed by UV illumination after electrophoresis on an ethidium bromide-stained 3% (w/v) agarose gel (NuSieve[®]; FMC BioProducts, Rockland, ME) run at 8 V cm^{-1} for 30–45 min. Control samples were taken from embryo washing media and subjected to the same treatments as embryos.

Estimation of parthenogenetic activation

Since parthenogenetically activated oocytes are diagnosed as females, they represent a potential source of bias in assessing female embryo growth. Therefore some oocytes were treated as above but inseminated with dead spermatozoa to estimate the relative proportion of cleavage of parthenogenetically activated oocytes in the two culture media. Spermatozoa were killed by repeated freezing–thawing in fert-TALP.

Statistical analysis

The effect of sex on the number of cells of embryos was determined by analysis of variance, using the general linear model (SAS Institute Inc., Cary, NC, USA). The effect of replicate was included in the model, since it was significant. Comparison of cleavage rates between treatments and comparison of sex ratios to the 1:1 ratio were carried out with the chi-squared test.

Results

Of the 11 replicates, three could not be used as a result of total PCR failure, probably owing to nuclease contamination of media used in zona pellucida removal. In the remaining eight replicates the cleavage rate was similar in glucose-free media and in media containing glucose (69.9% versus 63.8%; $P = 0.10$). Of the cleaved embryos, the sex of 91.3% could be determined. Of the embryos cultured without glucose, 100 were males and 108 were females. Embryos cultured in the presence of glucose included 91 males and 90 females. The sex ratio did not differ from the 1:1 ratio in either case.

The mean number of cells ($\pm$ SEM) was 5.07 ± 0.16 in culture without glucose and 5.29 ± 0.17 when glucose was present ($P = 0.86$). Without glucose in the medium, the least square mean was 0.22 cells higher in males compared with females. This difference was not significant ($P = 0.44$). However, in the presence of glucose, this difference was 1.25 cells in favour of males ($P = 0.0001$).

The incidence of parthenogenetic activation of oocytes inseminated with killed spermatozoa was 10 of 63 in the non-glucose and 8 of 63 in the glucose group ($P = 0.61$). At the end of culture, 15 of the 18 cleaved oocytes had developed to the two-cell stage. Of the remaining three parthenogenotes, two had three cells and one had four cells.

Discussion

The results presented here show that male embryos cleave faster than female embryos only in the presence of exogenous glucose. This is in agreement with our original assumption, and offers an explanation for previous observations of increased

development of male embryos. To our knowledge, sex differences in growth have not been observed following culture in media without glucose. Many of the authors who observed faster growth in males cultured their embryos in Menezo's B2 (Avery *et al.*, 1992; Marquant-LeGuienne *et al.*, 1992; Xu *et al.*, 1992), which contains a relatively high glucose concentration (6.7 mmol l^{-1}). Even when coculture with other cells is used with this medium, the amount of glucose would remain high, especially at the beginning of culture. Furthermore, Gutierrez *et al.* (1993) observed an altered sex ratio of mouse embryos cultured in BMOC (medium containing glucose) but not in CZB (medium without glucose). Although glucose is only one of the differences between these media, the altered sex ratio could be attributed to glucose present in BMOC.

We tested the effect of glucose between 24 and 48 h after insemination. At 24 h, the fertilized oocyte is at the S or G2 phase (Barnes and Eyestone, 1990). Our finding seems to rule out the possibility that earlier events (such as earlier fertilization or pronucleus formation) solely account for the faster development of males. However, we cannot exclude the possibility that sex differences established at earlier stages may be triggered by glucose 24 h after insemination.

It could be argued that males develop faster than females because the reduced DNA content would allow faster completion of DNA replication. However, the findings of Burgoyne (1993) do not support this. The increased growth in male embryos may well be a consequence of differential gene expression caused either by the Y chromosome or by the presence of a single X chromosome. While Burgoyne (1993) presents evidence for a Y-chromosomal effect in mice, our results in cows imply a role for an increased activity of the X-linked gene products HPRT and G6PDH in female embryos. Apparently both these gene products play a role in controlling the amount of oxygen radicals. The formation of inosinate from hypoxanthine is catalysed by HPRT, but Downs and Dow (1991) demonstrated that glucose partially inhibits the activity of HPRT. This could increase degradation of hypoxanthine to ureate in reactions generating H_2O_2 . Whereas the absence of HPRT inhibition may facilitate a decrease in peroxidation efficiently; even with single gene expression, a partial inhibition may require expression of both genes to do so. Therefore, it is possible that the difference in HPRT activity between the sexes can be expected to result in significantly different amounts of H_2O_2 only in the presence of glucose.

The activity of G6PDH controls the entry of glucose into the pentose phosphate pathway, which generates NADPH. With NADPH as electron source, glutathione-SH can reduce peroxides. The absence of exogenous glucose would most probably decrease the activity of the pentose phosphate pathway, which could be expected to diminish a sex-dependent difference in production of NADPH. High intracellular glucose utilization in the absence of exogenous glucose is unlikely. Khurana and Wales (1991) showed that glycogen degradation is inhibited in mouse embryos at a 20% oxygen concentration.

Consequently, increased gene expression of either HPRT or G6PDH may result in an abnormally low concentration of H_2O_2 in female embryos. The possibility that oxidative stress causes male embryos to develop faster than female embryos is supported by evidence of oxidation-induced gene expression (Sohal and Allen, 1990). For example, ADP-ribose transferase

activity is stimulated by oxidative stress (Cerutti, 1985) and is required for mitogen-induced activation of human lymphocytes (Johnstone and Williams, 1982). Furthermore, H_2O_2 induces chromosomal puffing in *Drosophila* (Compton and McCarthy, 1978) and redox balance influences translation of mRNA (Douzou and Maurel, 1977).

The concept that differential gene expression causes the observed growth difference rests upon the assumption that gene activity occurs before the eight-cell stage. Although the major surge of transcription in cow embryos is generally accepted to occur at the eight- to 16-cell stage, the data of Barnes and First (1991) suggest that the first transcription events occur at the five–six-cell stage or earlier. Plante *et al.* (1994), Marcucio *et al.* (1995) and Iwasaki *et al.* (1995) provided evidence of zygotic gene expression at the two-cell stage. It would make sense that housekeeping genes, such as those encoding HPRT and G6PDH, would be among the first to be activated.

Barnes and First (1991) and Plante *et al.* (1994) observed an increase in the growth rate of cow embryos exposed to the transcription inhibitor α -amanitin. This may at first appear surprising. However, according to our hypothesis, blocking transcription would increase intracellular H_2O_2 , and hence increase the growth rate. We intend to test our hypothesis further by examining the effect of blocking transcription on cow embryos cultured in glucose. The absence of the growth rate difference by the inhibition of transcription would be in agreement with our hypothesis.

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